Master's Thesis

Electro-Biocatalytic Conversion of CO_2 into Formic acid by Whole-cell and Immobilized Enzyme

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Approved by

Advisor
Yong Hwan Kim

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Abstract

Utilizing carbon dioxide to valuable chemicals is attractive technology for reducing CO_2 emission. Among the chemicals converted from CO_2, formic acid is one of the most valuable chemicals. Efficient conversion of CO_2 to formic acid by electro-biocatalytic system was reported without expensive cofactor and noble metals. In this study, *Shewanella oneidensis* MR-1 (*S. oneidensis* MR-1) and encapsulated Formate dehydrogenase1 from *Methylobacterium extorquens* AM1 (MeFDH1) were applied to electro-biocatalytic reaction as a whole cell and encapsulated biocatalyst, respectively.

First, *S. oneidensis* MR-1, when aerobically grown in Luria-Bertani (LB) medium, exhibited its ability for the conversion of CO_2 into formic acid with productivity of 0.59 $mM \cdot hr^{-1}$ for 24 hr. In addition, CO_2 reduction reaction catalyzed by *S. oneidensis* MR-1, when anaerobically grown in newly optimized LB medium supplemented with fumarate and nitrate, exhibited 3.2-fold higher productivity (1.9 $mM \cdot hr^{-1}$ for 72 hr).

Second, previous study has demonstrated that electro-biocatalytic conversion of CO_2 to formic acid by engineered MeFDH1 shows higher productivity than wild type. To increase physical strength, stability, reusability of MeFDH1, MeFDH1 was encapsulated in pure alginate and alginate silica hybrid beads. Michaelis-Menten kinetic constants demonstrated that binding affinity and maximum reaction rate of both encapsulated MeFDH1 were declined. Compared with pure alginate beads (5.4%), alginate-silica hybrid beads (67.4%) exhibited more higher recycling productivity after 4 cycles. These results show that the immobilization of MeFDH1 through encapsulation of by alginate-silica hybrid is a more suitable method to recycle formate production and prevent leakage of MeFDH1.

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Nomenclature

FDH: Formate dehydrogenase

S. oneidensis MR-1: *Shewanella oneidensis* MR-1

M. extorquens AM1: *Methylobacterium extorquens* AM1

MeFDH1: Formate dehydrogenase 1 from *Methylobacterium extorquens* AM1

MV: Methyl viologen

EV: Ethyl viologen

ALG: Calcium alginate beads

ALG-SiO_2: Calcium alginate silica hybrid beads

NADH: Reduced nicotinamide adenine dinucleotide

1. Introduction

1.1. Carbon dioxide capture and utilization

According to recent trend of world greenhouse gas emission, not only the total amount of greenhouse gases but also the proportion of CO_2 in greenhouse gases has been increasing consistently.[1] The relationship between global temperature and atmospheric CO_2 concentration clearly showed that CO_2 is a major cause of global warming.[2-3] Utilizing CO_2 captured from fossil power plant and other sources to convert to valuable chemicals is suggested as an attractive alternative to reduce CO_2 emissions.[4-5] Algae, building materials, fuels, polymer and chemical intermediates such as methanol, syngas and formic acid can be produced from captured CO_2. Among the chemicals synthesized from CO_2, formic acid is one of the products that have high potential to reduce CO_2 emissions economically.[6] Formic acid can be used as an energy carrier and more suitable as a chemical intermediate since it is more reactive than methanol.[5-7]

There are various researches to convert CO_2 to formic acid using chemical, electrochemical, photochemical and biological approaches.[8-12] Although various chemical catalysts have been reported for reduction reaction of CO_2 to formic acid, reduction reactions using chemical catalysts require harsh condition (high temperature and pressure) and noble metals (e.g. ruthenium, rhodium and iridium).[8-9, 13] In contrast, reduction of CO_2 to formic acid by biocatalytic reaction exhibit more mild condition and high selectivity.[8-9, 14-16]

Formate dehydrogenase (FDH) can catalyze reduction of CO_2 to formic acid without other organic chemicals. Enzymatic hydrogenation of CO_2 to formic acid via FDH has already been reported, but expensive hydrogen gas or NADH cofactor were required.[13, 15] Therefore, to overcome these issues, electro-biocatalytic approaches were applied. Instead of hydrogen gas and NADH, water electrolysis at the anode supplied both electrons and proton and then these electrons were mediated by methyl viologen carriers from electrode.[8, 17] Here, we report whole cell and immobilized enzyme as electro-biocatalysts for the synthesis of formic acid from CO_2.

1.2. Whole cell electro-biocatalyst

Shewanella oneidensis MR-1 (*S. oneidensis* MR-1) is a facultative aerobic gram-negative bacteria and is also known as a metal-reducing bacteria. *S. oneidensis* MR-1 has almost 41 putative c-type cytochromes for respiration. Outer membrane decaheme cytochromes (OmcA and OmcB also known as MtrC) exposed to extracellular environment, periplasmic cytochromes (MtrA), outer membrane protein (MtrB), inner membrane tetraheme cytochrome c protein (CymA) are crucial to metal

reduction.[18-20] The electrons are transported through respiratory pathways and quinone cycling from electron donor such as metal, lactate, pyruvate and formate.[21] In addition to the remarkable electron transfer system, *S. oneidensis* MR-1 also has three periplasmic FDHs.[22-23] With those attractive characteristics, *S. oneidensis* MR-1 was applied to synthesis of formic acid as a whole cell electro-biocatalyst. Moreover, the optimization in growth condition of S. oneidensis MR-1 for the high expression of FDHs and electron carrier proteins resulted in remarkable improvement in formic acid synthesis.

1.3. Formic acid synthesis by encapsulated MeFDH1

Electro-biocatalytic synthesis of formic acid using wild type *Methylobacterium extorquens* AM1 (*M. extroquens* AM1) has been reported.[8] Furthermore, engineered *M. extroquens* AM1 to overexpress FDH showed 2.5 times higher productivity of formic acid than that of wild type.[24] However, enzymatic reactions have several disadvantages, such as the high cost of enzyme production and the requirement of subsequential purification.[25-28]

Therefore, immobilization techniques for enzymes are widely used to increase physical strength, stability and reusability. Immobilization of enzyme can also improve economic operation ability, automation and purity of products.[29] Among various enzyme immobilization methods, encapsulation of enzyme using alginate is the most commonly used due to good biocompatibility, easy application, and low material cost.[26-27] However, encapsulation in pure calcium alginate beads showed that large pore size of the alginate beads structure led to high leakage of enzyme.[30-31] To decrease leakage and increase stability of enzyme, cross linking between silica and alginate was applied.[26] These application of silica-alginate hybrid beads to immobilize MeFDH1 for electro-biocatalytic formic acid synthesis showed reusability and also increase stability compared to pure alginate.

2. Results and discussion

2.1. Electro-biocatalytic reduction of CO_2 by whole-cell biocatalyst

2.1.1. Synthesis of formic acid catalyzed by aerobically grown *S. oneidensis* MR-1

To verify whether *Shewanella oneidensis* MR-1 (*S. oneidensis* MR-1) could function as a whole-cell biocatalyst for the electro-biocatalytic synthesis of formic acid from CO_2 in a cathode reactor, we first investigated aerobically grown *S. oneidensis* MR-1 in LB medium (wet-cell weight of 1.0 g). Other reaction conditions followed the optimal conditions suggested in our previous study catalyzed by *Methylobacterium extorquens* AM1 (*M. extorquens* AM1) (i.e., CO_2 purging with flow rate of 1 mL/sec and 10 mM methyl viologen (MV) as electron mediator in 0.2 M potassium phosphate buffer pH = 7.0).[8] *S. oneidensis* MR-1, when aerobically grown in LB medium, exhibited an ability to covert CO_2 into formic acid, as shown in Figure 1. The control experiments (i.e., in the absence of *S. oneidensis* MR-1 or MV) did not produce any detectable formic acid (Table 1). *S. oneidensis* MR-1 was already known for its direct electron transfer ability.[32] However, the electrical current that was generated by *S. oneidensis* MR-1 was too low to produce detectable formate in the absence of the electron mediator, MV (Table 1). These control experiments confirmed that the formic acid was only produced from CO_2 catalyzed by *S. oneidensis* MR-1 whole-cell with MV as an electron mediator. Figure 1 shows that formic acid was produced relatively fast within 16 hr and then gradually increased to a saturated concentration of 14.1 mM after 24 hr. The titer of formic acid after a 20 hr reaction catalyzed by 1.0 g *S. oneidensis* MR-1 was 12.6 mM. The LB medium or aerobic condition might not be suitable to the overexpression of formate dehydrogenases of *S. oneidensis*. To further increase in reaction rate and titer of formic acid, the optimization of the growth conditions of *S. oneidensis* MR-1 was done and is presented in the next section.

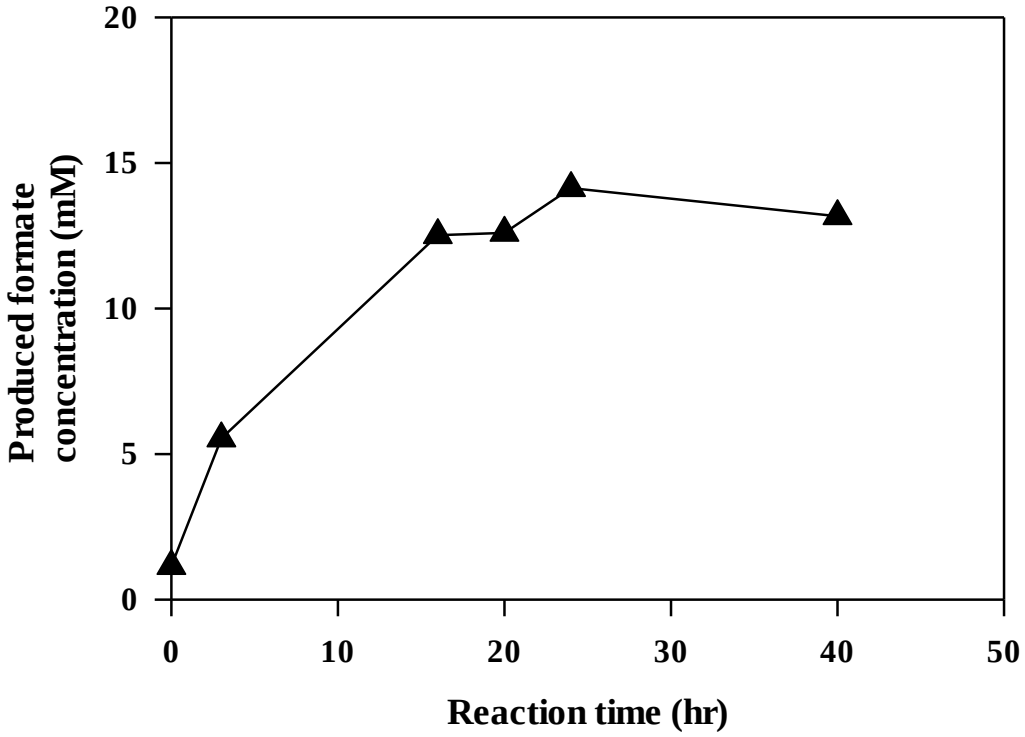

Figure 1. The synthesis of formic acid from CO_2 reduction with 10 mM MV catalyzed by *S. oneidensis* MR-1 (wet-cell weight of 1.0 g) which were aerobically grown in LB as whole-cell biocatalysts at pH = 7.0.

Table 1. Formic acid production by various conditions of electro-biocatalytic catalysis

Reaction condition	#1	#2	#3	#4	#5	#6	#7	#8
Shewanella oneidensis MR-1 (0.5 g wet-cell weight)	+	+	+	+	-	-	-	-
10 mM Methyl viologen (electron mediator)	+	-	+	-	+	-	+	-
Potential (-0.75V vs Ag/AgCl)	+	+	-	-	+	+	-	-
CO_2 gas purging	+	+	+	+	+	+	+	+
Formic acid production after 6 hr (mM)	25.9	ND	ND	ND	ND	ND	ND	ND

2.1.2. Optimization of growth conditions of *S. oneidensis* MR-1 for formic acid synthesis

The genome sequence of *S. oneidensis* MR-1 showed that three formate dehydrogenases (FDHs) (i.e., the genes encoding for those FDHs are fdnGHI [SO_0101 to SO_0103], fdhA1B1C1 [SO_4508 to SO_4511], and fdhA2B2C2 [SO_4512 to SO_4515]) existed in *S. oneidensis*.[20] Gene expression profiles have shown that abundance of mRNA for two formate dehydrogenases (i.e., selenocysteine-dependent and cytochrome b556-dependent formate dehydrogenases) in *S. oneidensis* MR-1 was significantly increased in the presence of several electron acceptors (i.e., fumarate or nitrate) under anaerobic conditions compared to in the absence of those acceptors under aerobic condition. In addition, the mRNA levels for genes related to formate degradation were not changed.[33] In other words, the presence of fumarate or nitrate as electron acceptors in the growth medium of *S. oneidensis* MR-1 under anaerobic conditions would be beneficial for overexpression of FDHs.

2.1.2.a. Effect of fumarate

The effect of fumarate in growth medium of *S. oneidensis* MR-1 on formic acid synthesis was determined by the addition of various concentrations of fumarate (10 ~ 60 mM) to LB-lactate medium (i.e., LB medium supplemented with 20 mM DL-lactate as an electron donor). The *S. oneidensis* MR-1, which was anaerobically grown in LB-lactate medium supplemented with various concentrations of fumarate, was used as a whole-cell biocatalyst for electro-biocatalytic synthesis of formic acid from CO_2. Addition of fumarate in growth medium of *S. oneidensis* MR-1 positively impacted the synthesis of formic acid (Figure 2). The increase in fumarate concentration (10 ~ 40 mM) in the growth medium of *S. oneidensis* MR-1 correlated to the increase in the reaction rate of formic acid catalyzed by the corresponding *S. oneidensis* MR-1 whole-cell biocatalysts (i.e., 11.5, 35.9 and 52.9 mM formic acid was synthesized after 33 hr, catalyzed by *S. oneidensis* MR-1 when anaerobically grown in the LB-lactate supplemented with 10, 20, and 40 mM fumarate, respectively). The reaction rate for synthesis of formic acid catalyzed by *S. oneidensis* MR-1 grown in LB-lactate medium supplemented with 60 mM fumarate was similar to that catalyzed by *S. oneidensis* MR-1 when grown in LB-lactate medium supplemented with 40 mM fumarate. In other words, the presence of fumarate (40 mM) in the growth medium enabled *S. oneidensis* MR-1 to almost reach its saturation point in the synthesis of formic acid.

As shown in Figure 2, the average reaction rate for formic acid synthesis within the first 24 hr of reaction catalyzed by *S. oneidensis* MR-1 when anaerobically grown in the LB-lactate medium supplemented with 40 mM fumarate was 5.9-fold faster than catalyzed by *S. oneidensis* MR-1 when aerobically grown in LB medium (i.e., 3.5 and 0.59 $mM \cdot hr^{-1} \cdot g^{-1}$ wet-cell, respectively). Importantly, the titer of formic acid catalyzed by *S. oneidensis* MR-1 when anaerobically grown in the LB-lactate

supplemented with 40 mM fumarate medium surpassed the saturated concentration of 14.1 mM catalyzed by *S. oneidensis* MR-1 when aerobically grown in LB medium and kept increasing constantly.

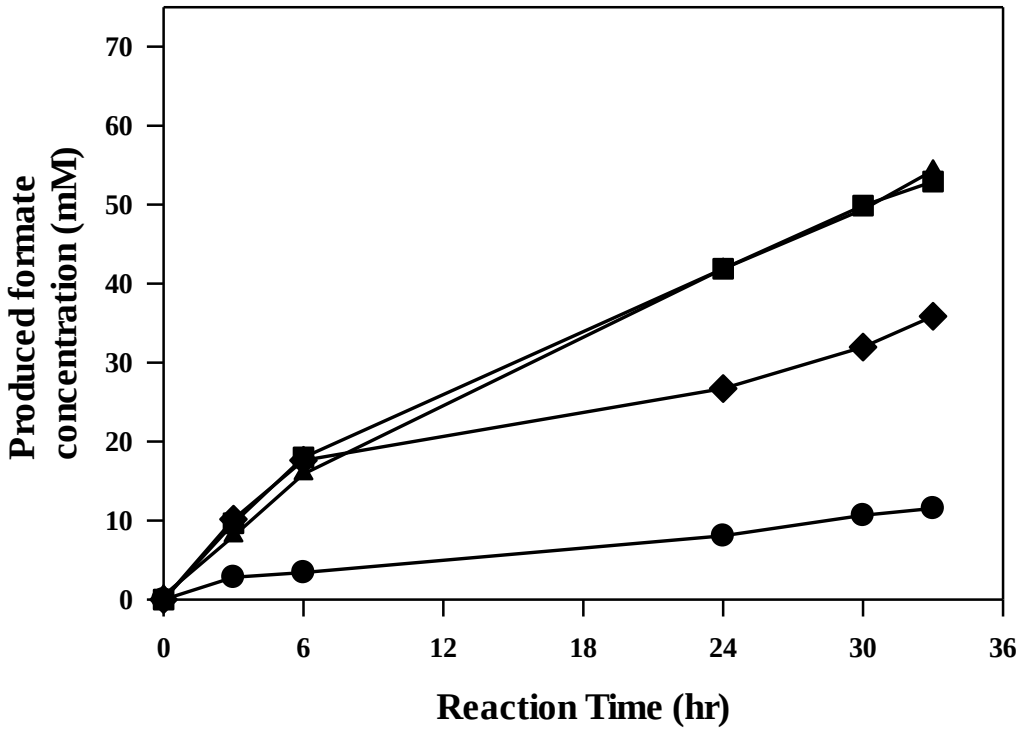

Figure 2. The formic acid synthesis from CO_2 reduction catalyzed by *S. oneidensis* MR-1 whole cells when grown in LB medium and 20 mM DL-lactate (LB-lactate) supplemented with various concentrations of Fumarate under anaerobic condition. A total of 0.4 g *S. oneidensis* MR-1 grown with 10 mM fumarate (●), 0.5 g *S. oneidensis* MR-1 grown with 20 mM fumarate (♦), 40 mM fumarate (■), 60 mM fumarate (▲).

2.1.2.b. Effect of nitrate and fumarate

It was found that nitrate positively impacted the abundance of mRNA for the two FDHs and several electron carrier proteins of *S. oneidensis* MR-1 under anaerobic conditions.[33] The effect of various concentrations of nitrate into the optimal LB-fumarate medium (i.e., LB supplemented with 40 mM fumarate and 20 mM DL-lactate) on the growth of *S. oneidensis* MR-1 as well as the production of formic acid catalyzed by *S. oneidensis* MR-1 when grown in the above medium was investigated.

The presence of nitrate in the optimal LB-fumarate medium of *S. oneidensis* MR-1 under anaerobic conditions strongly impacted the formic acid synthesis catalyzed by the corresponding *S. oneidensis* MR-1 whole-cell biocatalysts (Figure 3). The reaction rate of formic acid synthesis catalyzed by *S. oneidensis* MR-1 when anaerobically grown in the optimal LB-fumarate medium supplemented with 1 mM nitrate (the optimal LB-fumarate-nitrate concentration) was significantly improved compared to that catalyzed by *S. oneidensis* MR-1 when anaerobically grown in the optimal LB-fumarate in the absence of nitrate. Remarkably, 136.8 mM formic acid was produced from the CO_2 reduction catalyzed by *S. oneidensis* MR-1 grown in optimal LB-fumarate supplemented with 1 mM nitrate after 72 hr, as shown in Figure 3. The average conversion rate to formic acid catalyzed by *S. oneidensis* MR-1 when anaerobically grown in the optimal LB-fumarate medium within 72 hr reactions in the presence and absence of 1 mM nitrate were 3.8 and 1.8 $mM \cdot hr^{-1} \cdot g^{-1}$ wet-cell, respectively.

As seen in Figure 3 and 4, the presence of higher concentrations of nitrate (5 mM or 10 mM) in the optimal LB-fumarate medium extremely impacted on the growth rate of *S. oneidensis* MR-1 as well as the formic acid synthesis catalyzed by *S. oneidensis* MR-1. It has been shown that nitrate strongly inhibited fumarate chemotaxis of *Shewanella putrefaciens* MR-1 (the old name of *Shewanella oneidensis* MR-1).[34] The presence of a high concentration of nitrate in the optimal LB-fumarate might negatively impact *S. oneidensis* MR-1 metabolism as well as the expression levels of FDHs in *S. oneidensis* MR-1.

As seen in Figure 3, the titer of formic acid from CO_2 reduction catalyzed by the *S. oneidensis* MR-1 when grown in the optimal LB-fumarate supplemented with 1 mM nitrate reached the maximal concentration of 136.8 mM after 72 hr and then slightly decreased to 125.5 mM after 96 hr. Formic acid synthesis from hydrogenation of CO_2 and H_2 catalyzed by the *Acetobacterium woodii* whole-cell biocatalyst also reached a saturated concentration at an early stage of reaction due to reaction pH.[15] The pH the reaction solution catalyzed by *S. oneidensis* MR-1 when anaerobically grown in optimal LB-fumarate supplemented with 1 mM nitrate dropped to approximately 5.0 after 96 hr. It was reported that at pH values below 6.5 the absorption efficiency of the MV radical decreased significantly.[35] The reaction solution at a low pH (~5.0) within period of 72 or 96 hr would be the

reason for the instability of MV and the subsequent decrease in the titer of formic acid. Neutralizing the reaction solution during the reaction through a pH control system or an anionic exchange resin for the adsorption of produced formic acid would be beneficial in increasing the titer of formic acid.

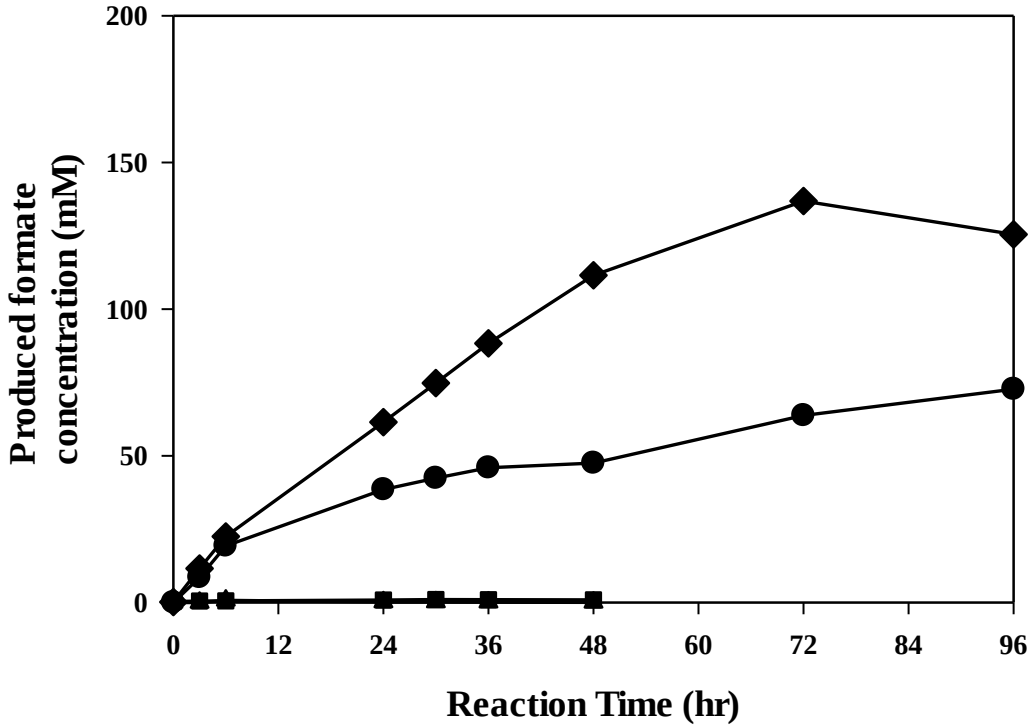

Figure 3. The formic acid synthesis from CO_2 reduction catalyzed by *S. oneidensis* MR-1 whole cells when grown in the optimal LB- fumarate medium (LB supplemented 40 mM Fumarate and 20 mM DL-Lactate) supplemented with various concentrations of nitrate under anaerobic condition. A total of 0.5 g *S. oneidensis* MR-1 grown without nitrate (●), with 1 mM nitrate (♦), 0.3 g *S. oneidensis* MR-1 grown with 5 mM nitrate (■), with 10 mM nitrate (▲).

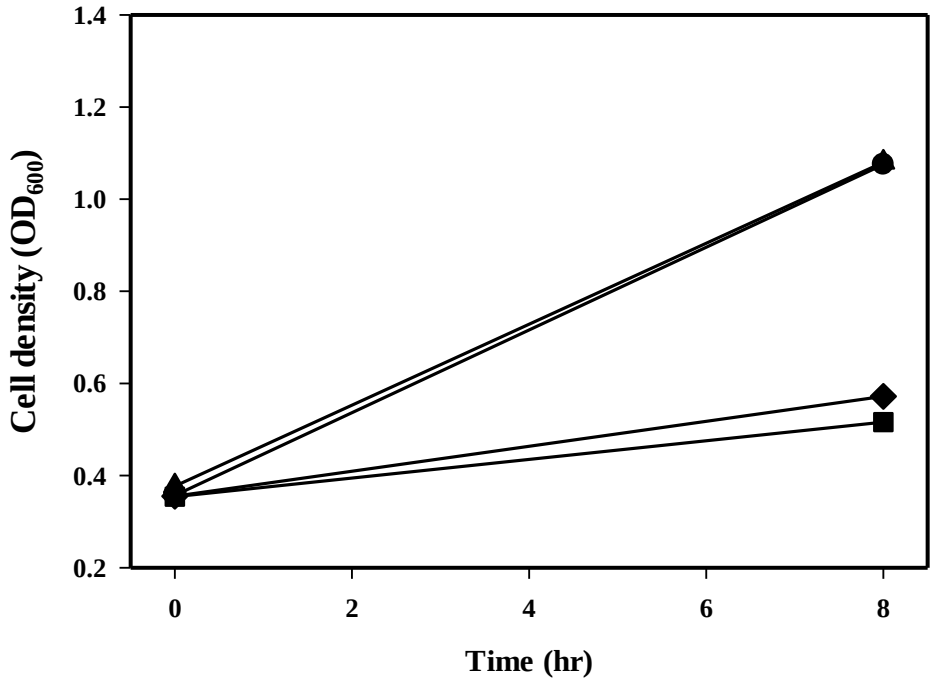

Figure 4. The growth of *S. oneidensis* MR-1 under anaerobic condition in the optimal LB- fumarate medium (i.e., LB medium supplemented with 40 mM Fumarate and 20 mM DL-lactate) supplemented with various concentrations of nitrate. grown without nitrate (●): wet-cell weight 1.711 g/L, with 1 mM nitrate (♦): 1.811 g/L, 5 mM nitrate (■): 0.694 g/L, 10 mM nitrate (▲): 0.701 g/L.

2.2. Encapsulated MeFDH1 for electro-biocatalytic conversion of CO_2

2.2.1. Effect of alginate concentration

Previous study in our lab with engineered *M. extorquens* AM1 to overexpress *Methylobacterium extorquens* AM1 formate dehydrogenase 1 (MeFDH1) showed higher formate productivity than wild type and demonstrated MeFDH1 is the crucial enzyme to convert CO_2 to formic acid.[24] Therefore, MeFDH1 was selected as an encapsulated biocatalyst for electrochemical reduction of CO_2 to formic acid. To increase physical strength, stability and reusability of MeFDH1, MeFDH1 was encapsulated in calcium alginate beads. For electrochemical reduction of CO_2 to formic acid, two substrates (CO_2 and ethyl viologen) should be bound to MeFDH1. However, diffusion rate of substrate through calcium alginate beads can be varied by calcium alginate concentration.[36-37] Purified MeFDH1 was encapsulated in various concentrations of alginate (0.5 ~ 2.0 %) beads to compare diffusion rate of substrates. The productivity of formic acid by encapsulated MeFDH1 using various alginate concentration was almost similar. Figure 5 shows that diffusion rate of CO_2 and ethyl viologen (EV) were not critically affected by alginate concentration. Other studies about the effect of alginate concentration on encapsulation efficiency shows that too low concentration of alginate exhibits a low physical strength and immobilization ratio due to the large pore size, and more than 2% alginate exhibits low enzyme activity due to small pore size.[38-39] Therefore, 2 % of alginate was used for encapsulation of MeFDH1 in further study

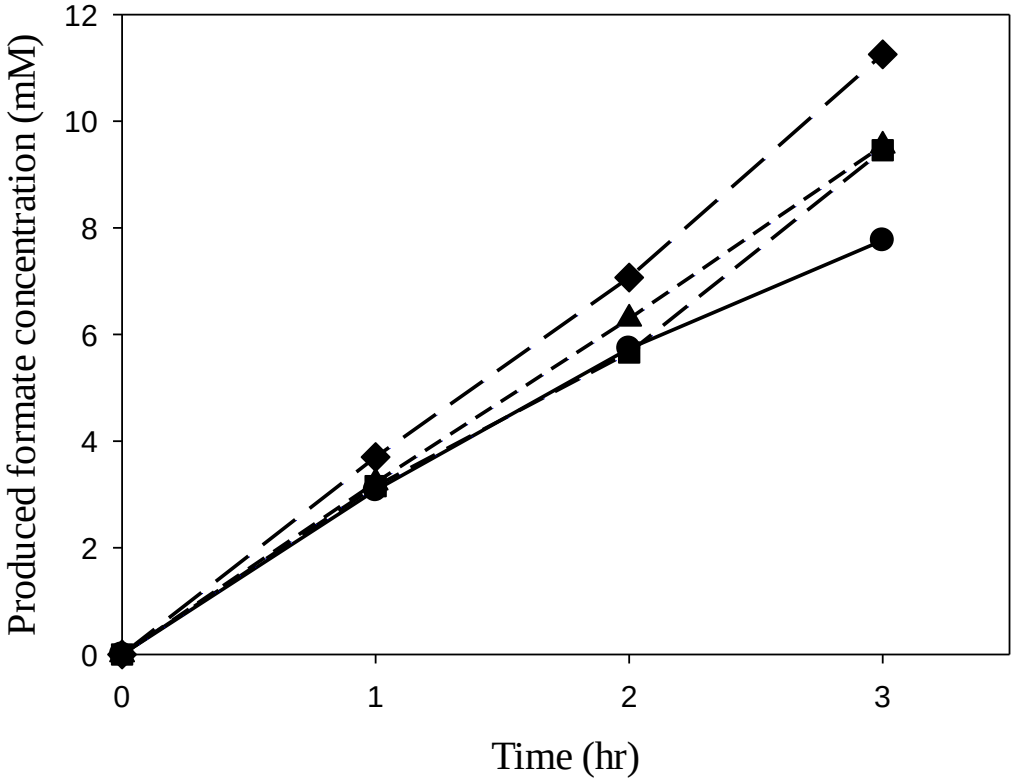

Figure 5. The synthesis of formic acid from CO_2 catalyzed by encapsulated MeFDH1 in various concentration of calcium alginate beads with 10mM EV at pH=7.0. MeFDH1 was encapsulated with 0.5 % alginate(●), 1 % alginate(♦), 1.5 % alginate(■), 2.0 % alginate(▲).

2.2.2. Kinetics of encapsulated MeFDH1

The activity of encapsulated enzyme is mostly affected by internal mass transfer limitations.[40] The Michaelis-Menten model was used to determine the kinetic parameters of the encapsulated MeFDH1. MeFDH1 catalyzes both formate oxidation and CO_2 reduction. In CO_2 reduction reaction, bicarbonate (dissolved form of CO_2) and reduced EV (EV^+) were used as substrate. However, the concentration of EV^+ for the Michaelis-Menten kinetics was too higher than suitable EV^+ allowing reliable absorbance range (absorbance of 0.1 mM EV^+ is almost 1.0).

Therefore, formate oxidation reaction is used to determine the Michaelis-Menten kinetic parameters. Formate and oxidized EV (EV^{2+}) were used as substrate and the reaction rates of formate oxidation reaction depending on the concentration of EV^{2+} and formate are shown in Figure 6 and 7, respectively. Lineweaver-Burk plots of encapsulated MeFDH1 were obtained from reaction rate depending on concentration of substrate (Figure 8, 9).

The Michaelis-Menten constant (K_M) for EV^{2+} of encapsulated MeFDH1 is 4.74 mM (pure calcium alginate) and 2.43 mM (alginate silica hybrid beads) respectively (Figure 10). These values are 11 and 5.9-fold higher than free MeFDH1 (K_M value of free MeFDH1 is not shown). K_M value for formate of encapsulated MeFDH1 is 5.25 mM (pure calcium alginate) and 4.35 mM (alginate-silica hybrid bead) respectively (Figure 10). K_M values for formate are also 3.4 and 2.9-fold higher than free MeFDH1 (K_M value of free MeFDH1 is not shown). The V_m values are 181.8 μM/min (pure calcium alginate), 67.6 μM/min (alginate-silica hybrid bead) for EV^{2+} and 357.1 μM/min (pure calcium alginate), 64.9 μM/min (alginate-silica hybrid bead) for formate (Figure 10). V_m values are also lower than free MeFDH1 (not shown), these results show that the binding affinity as well as the maximum reaction rate of MeFDH1 are declined by encapsulation.

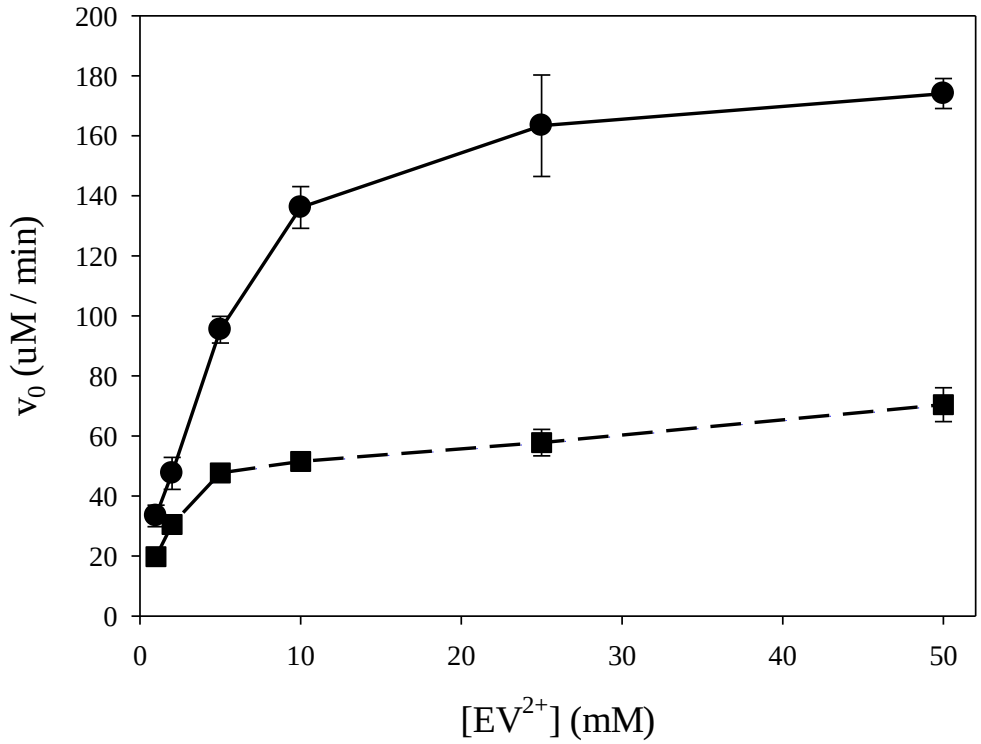

Figure 6. Reaction rate of formate oxidation reaction depend on the concentration of EV^{2+} by MeFDH1 encapsulated in pure alginate(●) and alginate-silica hybrid bead(■).

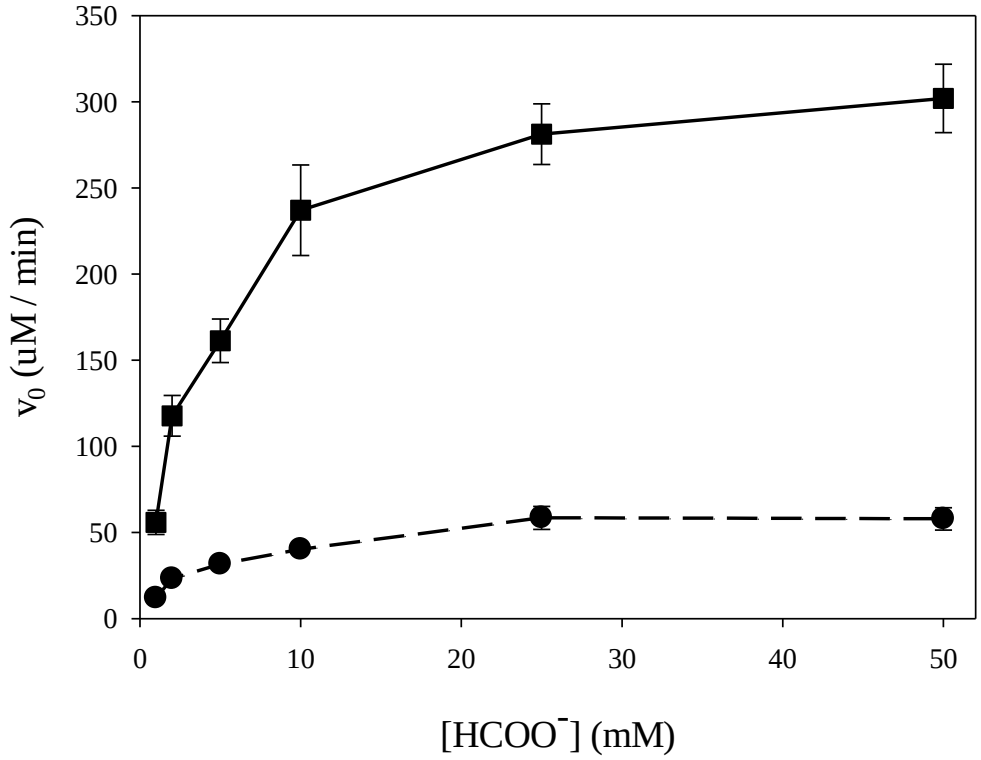

Figure 7. Reaction rate of formate oxidation reaction depend on the concentration of formate by MeFDH1 encapsulated in pure alginate(●) and alginate-silica hybrid bead(■).

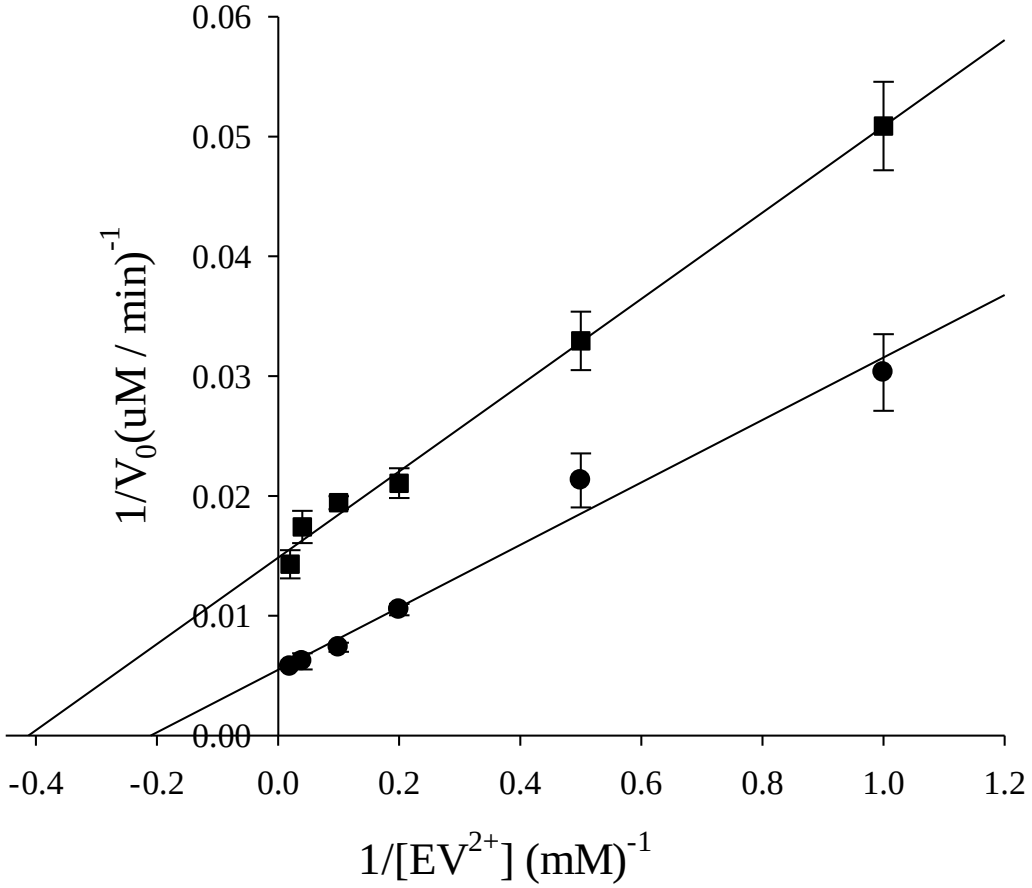

Figure 8. Lineweaver-Burk plots of encapsulated MeFDH1 by calcium alginate beads (●) and alginate-silica hybrid beads (■) using EV^{2+} as a substrate

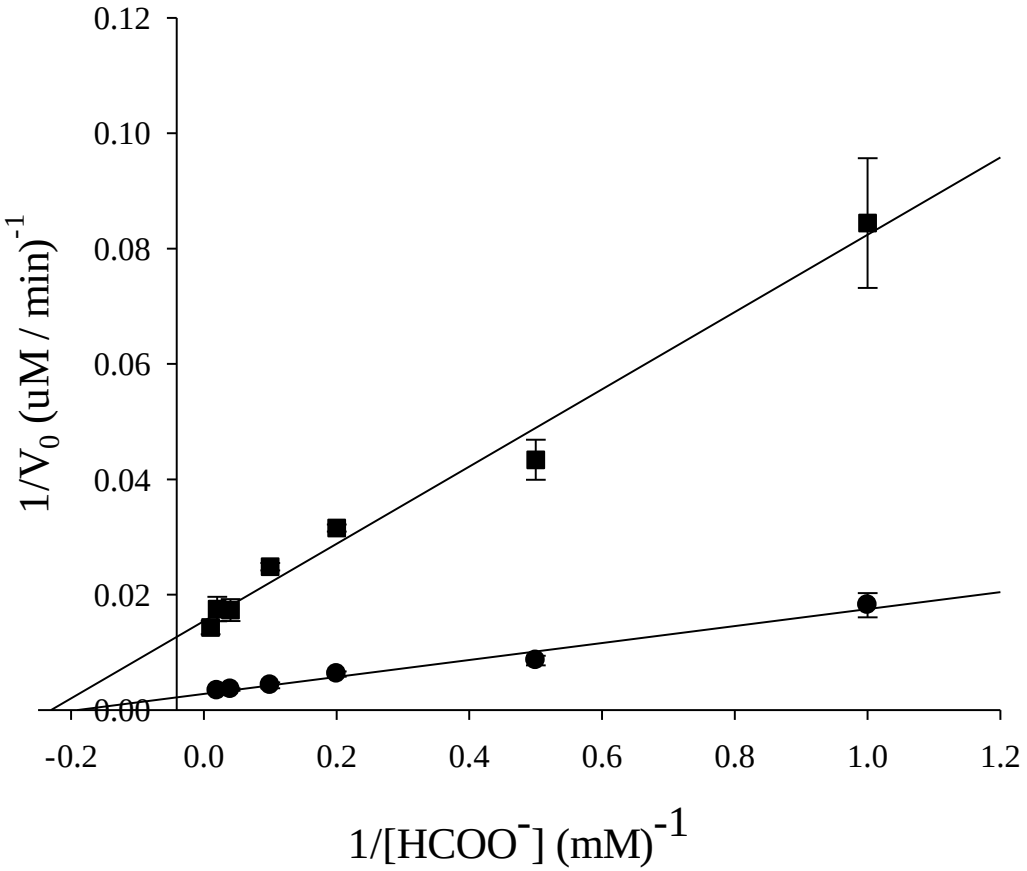

Figure 9. Lineweaver-Burk plots of encapsulated MeFDH1 by calcium alginate beads (●) and alginate-silica hybrid beads (■) using formate as a substrate.

Table 2. Kinetics constant of encapsulated MeFDH1

Substrate	Pure ALG-MeFDH1		ALG-SiO_2-MeFDH1	
	V_m μM/min	K_M mM	V_m μM/min	K_M mM
EV^{2+}	181.8	4.74	67.6	2.43
$HCOO^-$	357.1	5.25	64.9	4.35

2.2.3. Reusability of encapsulated MeFDH1

One of the main advantages of encapsulation is the reusablity of enzyme. The encapsulated enzyme can be easily seperated from reaction solution and reused. Encapsulated MeFDH1 was used for electro-biocatalytic reaction and reused every 3 hr. The formate productivity after first cycle approached 100 % compared with initial productivity. As shown in Figure 10, both encapsulated MeFDH1 in pure calcium alginate and algiante-silica hybrid bead shows reusability. However, formate productivity of MeFDH1 encapsulated in pure calcium alginate was reduced to 5.5 % after 4 cycles, while that encapsulated in algiante-silica hybrid bead was reduced to only 67 % (Figure 11). As shown in Figure 12, Pure alginate beads show leakage of MeFDH1 after 3hr, while leakage of MeFDH1 encapsulated in ALG-SiO2 beads wasn't observed. This results shows that encapsulation by algiante-silica hybrid bead is more suitable to maintain productivity as well as prevent leakage of MeFDH1 than pure alginate bead

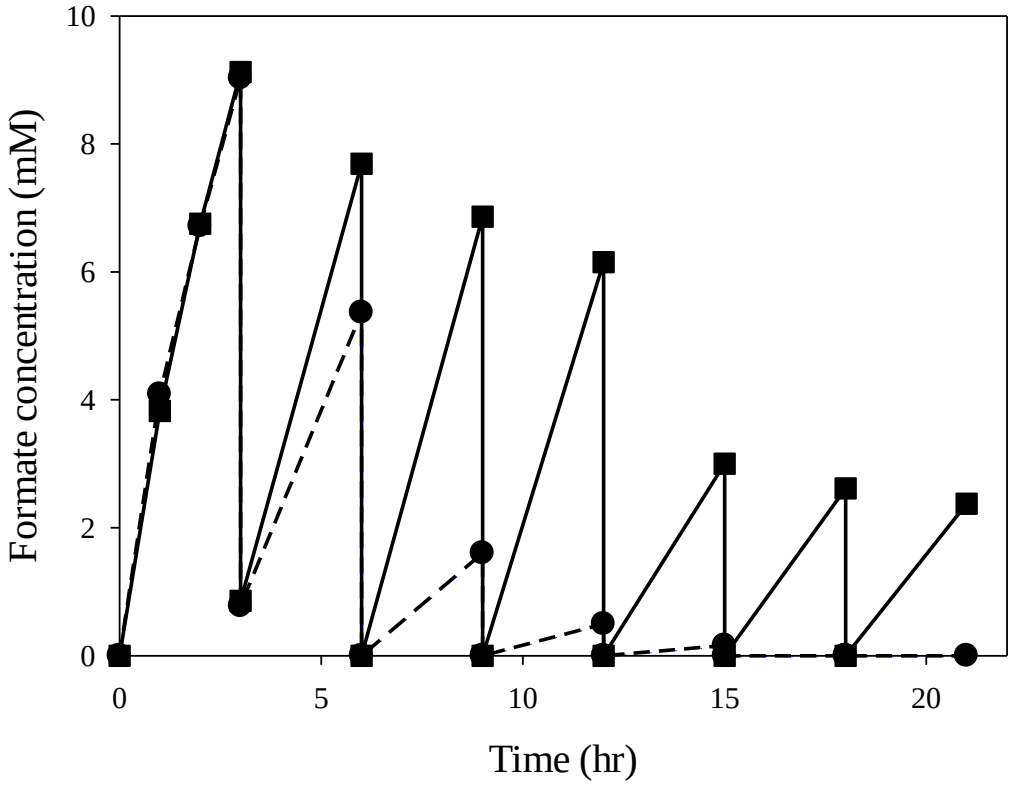

Figure 10. The reusability of encapsulated MeFDH1 in pure calcium alginate beads (●) and silica-alginate hybrid beads (■) with 10mM EV.

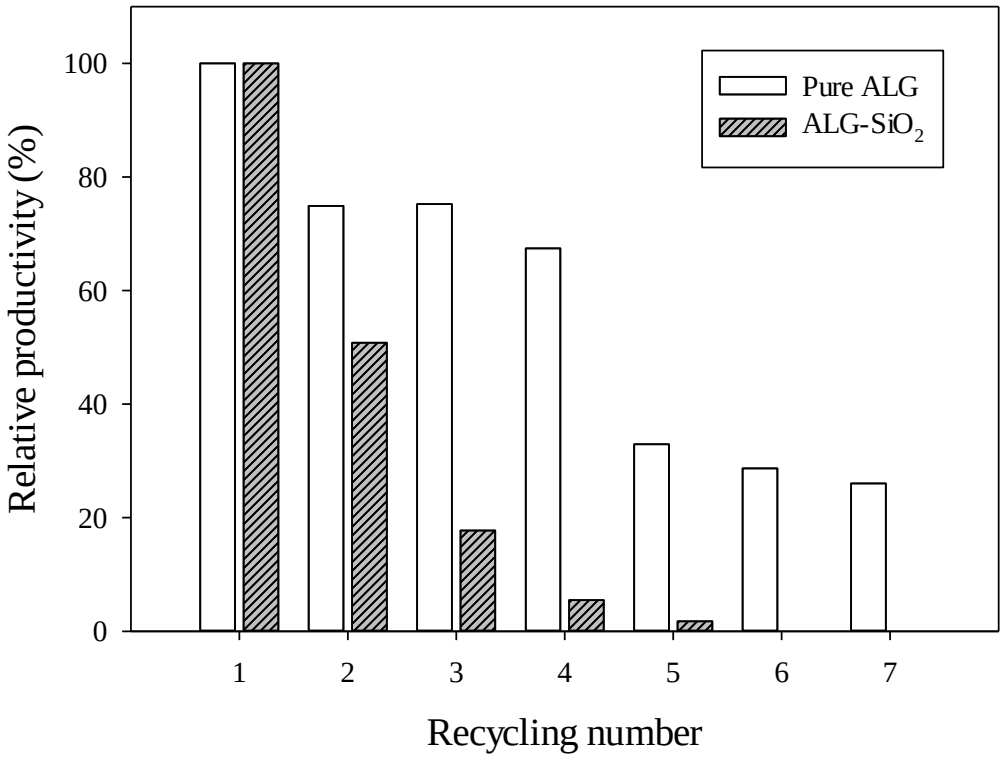

Figure 11. Relative formic acid productivity of encapsulated MeFDH1 in pure calcium alginate beads(pure ALG) and silica-alginate hybrid beads (ALG-SiO_2). The formate productivity of first cycle was taken to be 100 %.

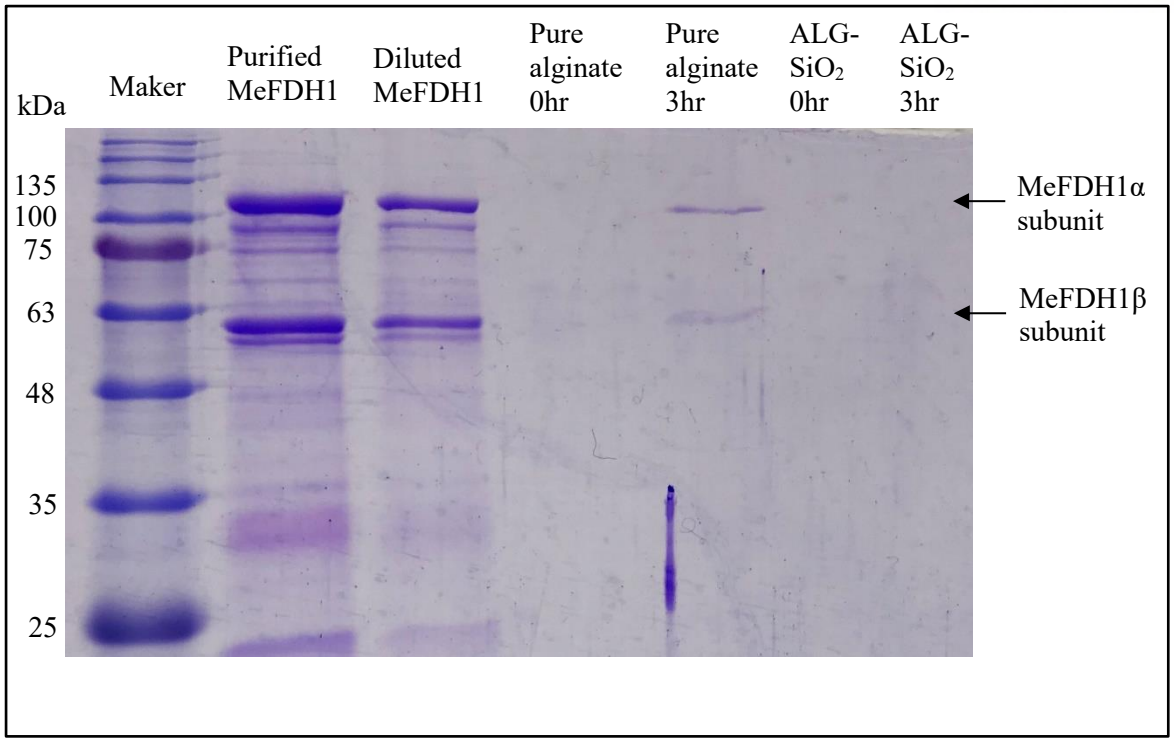

Figure 12. SDS-PAGE of MeFDH1 encapsulated in pure alginate and alginate-silica hybrid beads.

3. Conclusion

Electro-biocatalytic system was applied to efficiently convert CO_2 to formic acid by *Shewanella oneidensis* MR-1 (*S. oneidensis* MR-1) and encapsulated *Methylobacterium extorquens* AM1 (MeFDH1).

S. oneidensis MR-1, as a whole cell biocatalyst, aerobically grown in LB medium could produce 14.1 mM formic acid after a 24 hr reaction. The optimization of the growth condition of *S. oneidensis* MR-1 was successful. As a result, the CO_2 reduction catalyzed by *S. oneidensis* MR-1 anaerobically grown in the newly optimized medium (i.e., LB supplemented with 40 mM fumarate, 1 mM Nitrate and 20 mM DL-lactate) produced up to 136.84 mM formic acid after 72 hr. In addition, the average reaction rate for formic acid synthesis catalyzed by *S. oneidensis* MR-1 within 72 hr under anaerobic conditions in the optimal medium was 3.8 $mM \cdot hr^{-1} \cdot g^{-1}$ wet-cell.

MeFDH1 was successfully encapsulated in pure alginate and alginate-silica hybrid beads. In formic acid oxdiation reaction, the binding affinity and maximum reaction rate of both pure alginate and alginate-silica hybrid beads containing MeFDH1 tended to decrease. The alginate-silica hybrid beads maintained formate productivity of 67 %, whereas pure alginate beads decrease to 5.5 % after 4 cycles.

4. Materials and methods

4.1. Materials

Shewanella oneidensis MR-1 *(S. oneidensis* MR-1, ATCC®700550™) was purchased from America Tissue Culture Collection. Luria-Bertani (LB) medium was purchased from Duchefa Biochemie (2031 BH Haarlem, Netherlands). Sodium formate (a standard), DL-lactate sodium, sodium nitrate, sodium fumarate, methyl viologen (MV), ethyl viologen (EV), sodium alginate, calcium chloride, tetramethyl orthosilicate (TMOS) and other chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, United States) and used without treatment. Culture medium for *Methylobacterium extorquens* AM1 (*M. extorquens* AM1) was described in our previous study.[24]

4.2. Growth and harvest of *S. oneidensis* MR-1

The *S. oneidensis* MR-1 was grown under both aerobic and anaerobic conditions similar to a previous study.[32] First, *S. oneidensis* MR-1 (3 mL) in LB medium was incubated in an overnight culture at 30 °C, 200 rpm. For aerobic growth, the above seed culture (3 mL) was inoculated into LB medium (100 mL) and incubated at 30 °C, 100 rpm for 24 hr. For anaerobic growth, the above seed culture (6 mL) was inoculated into LB medium (200 mL) in 1 L flask and incubated at 30 °C, 200 rpm until OD_{600} reached 1.0. Then, the scaled-up culture (140 mL) was inoculated into 350 mL of anaerobically prepared LB medium supplemented with DL-sodium lactate (20 mM) and sodium fumarate (0 ~ 60 mM) and/or sodium nitrate (0 ~ 10 mM) in a 500 mL serum vial sealed with a vial stopper and incubated at 30 °C, 100 rpm for 8 hr. The cells were harvested by centrifugation (12,000 rpm at 4 °C for 2 min) and then washed 3 times with 200 mM phosphate buffer (pH = 7.0).

4.3. Purification of MeFDH1

Culture condition for Engineered *M. extorquens* AM1 was described in our previous study.[24] Engineered *M. extorquens* AM1 was suspend in 50 mM MOPS (pH 7.0 adjusted by KOH) containing 20 mM Imidazole and lysed by sonication (Sonic & Materials Inc. VCX750) (40% amplitude, total 40min, pulse every 2 s for 20min). Lysate was centrifuged to remove cell debris (12000 rpm for 20min). The Ni-NTA resin was packed into column and then the supernatant was loaded. The resin was washed with 10 column volumes of same MOPS buffer and eluted with same MOPS buffer containing 300 mM Imidazole. The concentration of purified MeFDH1 was measured using Nanodrop (Thermo Fisher Scientific, Nanodrop one) (Extinction coefficient of MeFDH1: 8.94).

4.4. Specific activity assays

The formate oxidation specific activity of purified MeFDH1 was measured with 50 mM MOPS (pH 7.0 adjusted by KOH) containing 30 mM potassium formate, 0.5 mM NAD^+. The reaction was initiated by adding 20 ng of MeFDH1 at 30℃. The activity was calculated by increased slope of NADH absorbance at 340 nm using UV-spectrophotometer (ε_{NADH} : 6220 $M^{-1}\cdot cm^{-1}$).

4.5. Encapsulation procedures of MeFDH1

Pure calcium alginate beads containing MeFDH1 was prepared as following steps. 25U of MeFDH1 was mixed with sodium alginate solution (final 2 % conc.) and the mixture dropwise into 200 mM $CaCl_2$ solution. After 30 min calcium alginate beads containing MeFDH1 were collected by filtration and washed with distilled water three times. To prepare alginate-silica hybrid beads, 0.7 ml of TMOS was mixed with sodium alginate solution (final 2 % conc.) and then 25 U of MeFDH1 was added. The rest procedure was same with pure alginate.

4.6. Kinetics parameters of encapsulated MeFDH1

The Michaelis-Menten kinetics constant was calculated by increased absorbance of EV^+ at 600 nm using UV-spectrophotometer (ε_{EV^+} : 10000 $M^{-1}\cdot cm^{-1}$). 0.1 mg of MeFDH1 encapsulated in pure alginate or alginate-silica hybrid beads was mixed with 50 mM MOPS (pH 7.0 adjusted by KOH) containing 100 mM formate (to measure activity depend on the concentration of EV^{2+}) or 50 mM EV^{2+} (to measure activity depend on the concentration of formate). After incubation for 30 min, reaction was initiated by adding various concentration of EV^{2+} (1 ~ 50mM) or formate (1 ~ 100mM). The absorbance of EV^+ was measured after 30 s (pure alginate) or 1 min (alginate-silica hybrid beads).

4.7. Electro-biocatalytic system for formic acid synthesis

The electro-biocatalytic reactor, electrodes, and experimental setup in this study were similar to our previous paper. 1.5 cm × 2.0 cm of copper plates (for *S. oneidensis* MR-1), carbon felt (for encapsulated MeFDH1) were used as the working electrode. Ag/AgCl electrode was used as the reference electrodes in the cathode section, while coiled platinum wire was utilized as counter electrodes in the anode section. The protons generated from the electrolysis of H_2SO_4 (1 mM for *S. oneidensis* MR-1, 10mM for encapsulated MeFDH1) by the anode were supplied to the cathode through a proton exchange membrane (Nafion 115, 1.0 cm ×1.5 cm). The experiments were conducted under potentiostatic control using MultiEmStat (PalmSens BV, Houten, The Netherlands)

and Multitrace software control. The cathode was polarized at -0.75 vs. Ag/AgCl reference electrode.[8] The current was recorded every 10 seconds for system control as well as Faraday efficiency analysis. The formic acid synthesis was performed in the cathode reactor by purging gaseous CO_2 (99.999% purity at flow rate of 1 ml/sec). 0.2 M potassium phosphate and 0.2 M MOPS buffer were used for *S.oneidensis* MR-1 and encapsulated MeFDH1 respectively (adjusted pH = 7.0 by KOH). Whole-cell *S. oneidensis* MR-1 (wet-cell weight of 0.5 g) and 10 mM methyl viologen (MV) as an electron mediator were added to the above reaction solution to final volume of 10 mL. In case of encapsulated MeFDH1, encapsulated MeFDH1 and 10 mM ethyl viologen (EV) were added to reaction buffer to final volume of 10 ml. The reaction was performed at room temperature (~25 °C) and stirred at 300 rpm.

The synthesized formate was analyzed by HPLC (i.e., Agilent 1200 series with Refractive Index detector (RID) and Rezex ROA-Organic Acid H^+ (8%) column using 5 mM H_2SO_4 (0.6 mL/min) as the mobile phase. To prepare the sample for HPLC analysis, 15 μL of H_2SO_4 (6 M) was added to reaction sample (150 μL) to quench the reaction, which was then centrifuged at 10,000 rpm for 1 minute to remove cell pellet. The broth was further filtered with a 0.2 μm hydrophilic filter. For each analysis, 20 μL samples were injected and formate was detected at 13.8 min in our analysis conditions.

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